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Metabolite profiles of striped marsh frog (*Limnodynastes peronii*) larvae exposed to the anti-androgenic fungicides vinclozolin and propiconazole are consistent with altered steroidogenesis and oxidative stress

Steven D. Melvin[a,*], Frederic D.L. Leusch[a,b], Anthony R. Carroll[b]

[a] Australian Rivers Institute, Griffith University, Southport, QLD 4222, Australia
[b] Griffith School of Environment, Griffith University, Southport, QLD 4222, Australia

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ABSTRACT

Amphibians use wetlands in urban and agricultural landscapes for breeding, growth and development. Fungicides and other pesticides used in these areas have therefore been identified as potential threats that could contribute towards amphibian population declines. However, relatively little is known about how such chemicals influence sensitive early life-stages or how short episodic exposures influence sub-lethal physiological and metabolic pathways. The present study applied untargeted metabolomics to evaluate effects in early post-hatch amphibian larvae exposed to the anti-androgenic fungicides vinclozolin and propiconazole. Recently hatched (Gosner developmental stage 25) striped marsh frog (*Limnodynastes peronii*) larvae were exposed for 96 h to vinclozolin at 17.5, 174.8 and 1748.6 nM and propiconazole at 5.8, 58.4 and 584.4 nM. Nuclear Magnetic Resonance (NMR) spectroscopy was performed on polar metabolites obtained from whole-body extracts. Both fungicides altered metabolite profiles compared to control animals at all concentrations tested, and there were notable differences between the two chemicals. Overall responses were consistent with altered steroidogenesis and/or cholesterol metabolism, with inconsistent responses between the two fungicides likely reflecting minor differences in the mechanisms of action of these chemicals. Broad down-regulation of the tricarboxylic acid (TCA) cycle was also observed and is indicative of oxidative stress. Interestingly, formic acid was significantly increased in larvae exposed to vinclozolin but not propiconazole, suggesting this metabolite may serve as a useful biomarker of exposure to androgen-receptor binding anti-androgenic contaminants. This study demonstrates the power of untargeted metabolomics for distinguishing between similarly acting, but distinct, pollutants and for unraveling non-endocrine responses resulting from exposure to known endocrine active contaminants.

1. Introduction

Amphibians commonly use wetlands in urban and agricultural landscapes as breeding grounds and habitat for larval growth and development (Hartel et al., 2013; Knutson et al., 2004; Mann et al., 2009). Fungicides and other pesticides used in these settings have consequently been identified as factors that may pose a threat to amphibian populations and could contribute to the reported global population declines (Brühl et al., 2011, 2013; Mann et al., 2009; Monastersky, 2014). Early amphibian life-stages are often particularly sensitive to chemical insult (Edginton et al., 2004; Ortiz-Santaliestra et al., 2006; Pauli et al., 1999) due to the permeability of larval skin and their reliance on water for gas exchange and the maintenance of osmotic balances (Berrill and Bertram, 1997; Berrill et al., 1993). Despite this, we know very little about how agricultural pesticides impact physiological parameters and processes in larval amphibians (Broomhall, 2005). This represents an important knowledge gap considering the threatened status of many amphibian populations (Monastersky, 2014) and the continual expansion of agricultural activities necessary to meet global food demands (Tilman et al., 2011).

Untargeted metabolomics offers a powerful approach to broadly explore how pollutants influence physiological and metabolic endpoints in wildlife (Oliveira et al., 2016). Nuclear magnetic resonance (NMR) spectroscopy is a convenient option for metabolite profiling due to relatively simple sample processing and a high level of repeatability, and has proven very useful for applications in ecotoxicology (Emwas et al., 2013; Lankadurai et al., 2013; Viant et al., 2003). The majority of metabolomics studies in aquatic ecotoxicology have focused on fish or shellfish (Cappello et al., 2016; Cappello et al., 2017; Ekman et al., 2007; Viant et al., 2003), with few cases describing effects in

* Corresponding author.
E-mail address: s.melvin@griffith.edu.au (S.D. Melvin).

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amphibians. Those few studies with amphibians have revealed correlations between developmental endpoints and metabolite profiles in unexposed amphibian larvae (Ichu et al., 2014), and similarly in those exposed to chemical stressors (Melvin et al., 2017). However, exposure to pesticides is assumed to occur over relatively short timeframes in natural settings since this will intuitively coincide with episodic pulse applications. As such, two reasonable questions are 1) does short-term exposure causes detectable and distinctive effects on the metabolome of early life-stage amphibian larvae, and if so 2) do such effects translate into meaningful higher-level biological outcomes (e.g., altered growth, development or reproduction)? The present study addresses the first of these questions.

Vinclozolin and propiconazole are fungicides that have been used globally for applications ranging from horticulture of fruits and vegetables to the maintenance of turf grass. These chemicals are being phased out in some parts of the world but are still used in many locations (Kidd et al., 2013; Pallavi Srivastava, 2013). Maximal vinclozolin concentrations in natural aquatic matrices have been predicted to be as high as 52 µg/L (Steeger and Garber, 2009), but the limited available monitoring data suggests surface water concentrations closer to 0.5 µg/L (Readman et al., 1997; Tillmann et al., 2001). Expected environmental concentrations for propiconazole have been suggested to be as high as 80 µg/L (DeLorenzo et al., 2001) but monitoring data again reports conazole fungicides at much lower concentrations (< 0.1–7.7 µg/L) in aquatic environments (Haarstad, 2012; Wightwick et al., 2011). Both fungicides are considered anti-androgenic (Lor et al., 2015; Makynen et al., 2000; Skolness et al., 2013; van Ravenzwaay et al., 2013) and there is evidence that they also elicit oxidative stress (Bruno et al., 2009; Gazo et al., 2013; Radice et al., 1998; Tu et al., 2015). Due to substantial interest in endocrine disruption, studies assessing the toxic impacts of both fungicides have mainly focused on sexual development and related reproductive performance, and primarily in rodents and fish (Bayley et al., 2002; Gazo et al., 2013; Hotchkiss et al., 2003; Lor et al., 2015; Makynen et al., 2000; Nesnow et al., 2011). However, as indicated this fails to consider possible subtle biochemical effects that might occur during early developmental life-stages, and overlooks a major class of globally threatened vertebrate species.

We applied ^{1}H NMR spectroscopy to assess changes to global metabolite profiles in striped marsh frog (*Limnodynsastes peronii*) larvae exposed to environmentally relevant concentrations of the fungicides vinclozolin and propiconazole for 96 h during early development.

2. Materials and methods

2.1. Experimental animals

A single fertilized foam nest of the Australian striped marsh frog (*Limnodynastes peronii*) was collected from an ephemeral pond located in Elanora, Queensland, Australia. The nest was transported to the laboratory in water from the collection site and transferred to a 60 L aquaria filled with moderately hard test water (USEPA, 1994) until hatching. The Griffith University Animal Ethics Committee approved the work, according to the principles of the Australian Code for the Care and Use of Animals for Scientific Purposes (Protocol No. ENV/03/16/AEC).

2.2. Experimental protocol

Technical-grade vinclozolin (Cat. No. 45705) and propiconazole (Cat. No. 45642) were purchased from Sigma-Aldrich (Castle Hill, Australia). These were dissolved in methanol to create concentrated stock solutions, and administered at less than 0.003% the volume of experimental vessels to achieve target concentrations.

Tadpoles were allowed to hatch and were subsequently maintained until yolk sac and gills were absorbed. After hatching and yolk sac reabsorption (Gosner developmental stage 25; Gosner 1960), larvae were randomly transferred into individual 50 mL glass beakers filled with moderately hard test water, and each beaker received a single ration of 10 mg of Sera micron® fry food (Sera GmbH). There were nine replicate beakers each of control (water only) and solvent control (0.003% MeOH), and three concentrations of each chemical. Each replicate contained a single tadpole. The vinclozolin treatment was administered at 17.5, 174.8 and 1748.6 nM (equivalent to 5, 50 and 500 µg/L, respectively) and propiconazole at 5.8, 58.4 and 584.4 nM (equivalent to 2, 20 and 200 µg/L, respectively). Following 96 h exposure, larvae were euthanized in buffered 3-aminobenzoic acid ethyl ester (MS-222; Sigma Aldrich, Castle Hill, Australia). Wet weights (mg) were recorded, and animals were snap frozen on liquid nitrogen and stored at −80 °C for extraction of global (whole body) metabolites.

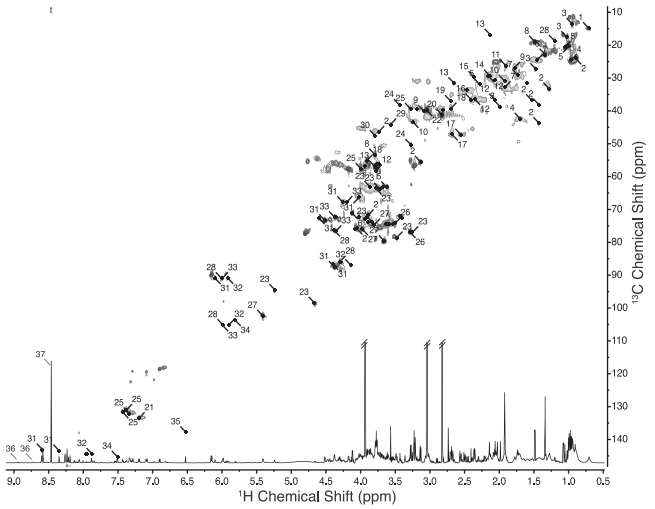

Fig. 1. HSQC spectra with water suppression showing annotated metabolites extracted from pooled sample of larval striped marsh frog (*Limnodynastes peronii*). 1 LDL, 2 Bile acids, 3 Isoleucine, 4 Leucine, 5 Valine, 6 Lactic acid, 7 Lysine, 8 Alanine, 9 Putrescine, 10 Arginine, 11 Acetate, 12 Glutamate, 13 Methionine, 14 Glutamine, 15 Pyruvic acid, 16 Succinic acid, 17 Citric acid, 18 Aspartate, 19 Dimethylamine, 20 Unkown metabolite, 21 Tyrosine, 22 Creatine, 23 Saccharides, 24 Taurine, 25 Phenylalanine, 26 *myo*-Inositol, 27 Glycogen, 28 UDP Glucose, 29 Glycine, 30 Guanidoacetic acid, 31 Nucleotide bases, 32 Uridine, 33 UDP Galactose, 34 Uracil, 35 Fumarate, 36 Niacinamide, 37 Formic acid.

2.3. Extraction of hepatic metabolites and lipids

Metabolites were extracted and separated from protein and lipids using the modified Bligh-Dyer methanol:chloroform extraction (Bligh and Dyer, 1959), as previously described (Lin et al., 2007; Melvin et al., 2017). Briefly, individual larvae were homogenized in 400 µg/L ice-cold methanol using an Ultra-Turrax® T10 tissue homogenizer (IKA®, Selangor, Malaysi) followed by ultra-sonication using a Q55 probe sonicator (Qsonica, USA), and incubated at −20 °C for 1 h. After incubation, 800 µL chloroform and 200 µL ultrapure water were added and the samples were vortexed and centrifuged (10 min, 16,000 × *g*, at 4 °C). The polar phase was carefully portioned into glass amber vials, the extraction was repeated with the remaining protein pellet, and the combined yields were stored at −80 °C until further processing.

2.4. ^{1}H NMR spectroscopy

Extracted metabolites were dried using a Series II centrifugal vacuum concentrator (GeneVac Technologies, England) and subsequently lyophilized. The metabolites were re-suspended in 200 µL phosphate buffer (K_2HPO_4 and NaH_2PO_4; pH 7.4) made with deuterium oxide (D_2O), which contained 0.05% sodium-3-(tri-methylsilyl)-2,2,3,3-